Received: 16 May 2023 | Revised: 12 January 2024 | Accepted: 11 February 2024

DOI: 10.1002/bmb.21825

ARTICLE

Biochemistry and Molecular Biology Education WILEY

Detection and enzymatic characterization of human saliva amylase

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Abstract

As a rule, an experiment carried out at school or in undergraduate study courses is rather simple and not very informative. However, when the experiments are to be performed using modern methods, they are often abstract and difficult to understand. Here, we describe a quick and simple experiment, namely the enzymatic characterization of ptyalin (human salivary amylase) using a starch degradation assay. With the experimental setup presented here, enzyme parameters, such as pH optimum, temperature optimum, chloride dependence, and sensitivity to certain chemicals can be easily determined. This experiment can serve as a good model for enzyme characterization in general, as modern methods usually follow the same principle: determination of the activity of the enzyme under different conditions. As different alleles occur in humans, a random selection of test subjects will be quite different with regard to ptyalin activities. Therefore, when the students measure their own ptyalin activity, significant differences will emerge, and this will give them an idea of the genetic diversity in human populations. The evaluation has shown that the pupils have gained a solid understanding of the topic through this experiment.

KEYWORDS

amylase, enzyme characterization, ptyalin

1 | INTRODUCTION

Modern bioanalytical methods are powerful and sophisticated. For students, however, they are often too abstract and difficult to understand. In the 1980s, for example, DNA sequencing provided autoradiograms, and in the 1990s, electropherograms, in which signal strength, background, and sample contamination could be seen directly. With next-generation sequencing, you put the ingredients together, press a button, and get a long list of sequences and quality parameters that only an expert can interpret. For undergraduate students, however, it is desirable to work with systems that provide unambiguous, clear, and descriptive results.

Here, we describe an example of an enzyme characterization experiment that is easy to perform and requires only basic laboratory equipment that is available even in high schools. The basic experiment can be varied to determine the pH or temperature optimum of the enzyme or to test its resistance to different chemicals.

Abbreviation: ddH_2O, double distilled water.

2 | BACKGROUND AND TEST PRINCIPLE

Alpha-amylase (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) digests both starch and glycogen by hydrolyzing α-1,4-glycoside bonds. The enzyme is active in a wide range of conditions; optimal conditions are pH 7.0, 30–40°C, and about 100 mM Cl^- (or Br^-). The enzyme produces the disaccharide maltose, which is split into two glucose molecules by the enzyme maltase in the course of the digestive process.

Human salivary amylase is ptyalin. This enzyme is robust and easy to obtain; untreated saliva can serve as a crude extract of ptyalin. Starch can be easily detected with Lugol's solution (aqueous I_2/KI solution), which is a simple detection method for the residual starch remaining after the incubation period with ptyalin.

Either continuous or discontinuous enzyme assays can be used for enzyme characterization. The former requires a measuring device capable of continuously recording data, but such devices are not available in most laboratories. Therefore, a discontinuous assay design has been chosen:

1. Saliva is taken as crude extract of ptyalin,
2. ... from which a dilution series is prepared.
3. An aliquot of each dilution stage is taken for a digestion batch with starch.
4. All reaction mixtures are incubated for the same length of time, and then the reactions are stopped with HCl.
5. Remaining starch is detected with Lugol's solution.

With this method, one or a few samples (with a high-saliva concentration) are obtained in which the starch has been completely broken down, one or a few (with a low-saliva concentration = high dilution of ptyalin) in which no starch has obviously been broken down, and one or two in the middle in which a small proportion of the starch has not yet been broken down, so that the iodine-starch test provides a weak (but clear) signal. Depending on the activity of the enzyme, this "tipping point" is reached at different dilutions. This experimental strategy enables a direct comparison of different reaction conditions, such as different pH values, temperatures, and so on.

3 | MATERIALS AND METHODS

Saliva (e.g., from pupils) is taken as a crude extract of ptyalin; it can simply be diluted with water. For the first dilution step, the students must be made aware of the viscosity of the saliva. It may be useful to use a pipette tip that is cut off at its end to enlarge the opening.

Table 1 shows the solutions required. Soluble starch according to Zulkowsky can be purchased as a substrate at any chemical store. The same applies to Lugol's solution and the other chemicals used.

Otherwise, only standard laboratory equipment is required: beakers or Petri dishes to collect the saliva, a laboratory balance, 15 or 50-mL plastic tubes for buffer and solution supplies, Eppendorf tubes for the saliva dilutions and reaction samples, piston-stroke pipettes (10, 100, and 1000 μL), and an incubator or oven (37°C). Students must wear safety goggles, especially when working with Lugol's solution and HCl (see below).

4 | THE BASIC TEST

For the basic test, a dilution series (dilution steps by a factor of three) is prepared in Eppendorf reaction vessels (Table 2). Now prepare and start seven reaction samples according to Table 3 at one-minute intervals. After 30 min of incubation at 37°C, the reactions are stopped with 100 μL of 1 M HCl, at one-minute intervals, too. To detect undegraded starch, 10 μL of Lugol's solution is added. At the end, the test is evaluated visually.

TABLE 1 Solutions.

Amylase (from saliva)	... diluted with ddH_2O (see Table 2)
Soluble starch according to Zulkowsky	2.4 g starch in 100 mL ddH_2O
Phosphate buffer pH 7.2 (10 mL)	2.8 mL 100 mM NaH_2PO_4 + 7.2 mL 100 mM Na_2HPO_4
NaCl	1 mL NaCl 0.5 M (in ddH_2O)
HCl	1 M
Lugol's solution	1% (w/v) I_2/2% (w/v) KI in ddH_2O

TABLE 2 Saliva dilution ("dS" = diluted sample).

dS.1	500 μL saliva +500 μL ddH_2O	=1:2 dilution
dS.2	300 μL dS.1 + 600 μL ddH_2O	=1:6 dilution
dS.3	300 μL dS.2 + 600 μL ddH_2O	=1:18 dilution
dS.4	300 μL dS.3 + 600 μL ddH_2O	=1:54 dilution
dS.5	300 μL dS.4 + 600 μL ddH_2O	=1:162 dilution

Note: To obtain a finer "scale," dilution steps by factor two (instead of three) can be chosen. In this case, however, two more dilution steps should be included to cover the same concentration range for ptyalin.

TABLE 3 Basic test setup.

Solution	R.P	R.1	R.2	R.3	R.4	R.5	R.N
PO_4 buffer pH 7.2[a]	300 µL	300 µL	300 µL	300 µL	300 µL	300 µL	300 µL
0.5 M NaCl[a]	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
Starch solution[a]	/	500 µL	500 µL	500 µL	500 µL	500 µL	500 µL
ddH_2O	500 µL	/	/	/	/	/	
100 µL aliquot from...[b,c]	dS.1	dS.1	dS.2	dS.3	dS.4	dS.5	
ddH_2O[c]							100 µL

Note: R.1–R.5: reaction samples. R.P, R.N: positive and negative control samples, respectively.
[a]See Table 1.
[b]See Table 2.
[c]Reactions are started with aliquots of diluted saliva (negative control: with ddH_2O) in one-minute-intervals.

TABLE 4 Buffers for different pH.

pH	Buffer components
3	238.4 µL 100 mM citric acid + 61.7 µL 100 mM NaH_2PO_4
5	145.5 µL 100 mM citric acid + 154.5 µL 100 mM NaH_2PO_4
6	263.1 µL 100 mM NaH_2PO_4 + 36.9 µL 100 mM Na_2HPO_4
7.2	84 µL 100 mM NaH_2PO_4 + 216 µL 100 mM Na_2HPO_4
8	15.9 µL 100 mM NaH_2PO_4 + 284.1 µL 100 mM Na_2HPO_4
9	4.6 µL 100 mM NaH_2PO_4 + 295.4 µL 100 mM Na_2HPO_4
11	283 µL 100 mM Na_2HPO_4 + 17 µL 100 mM Na_3PO_4

Note: Buffer capacity of the buffers at pH 9 and 11 is not very high which, however, is irrelevant when just pH dependency of the enzyme is to be visualized.

5 | TEST VARIATIONS

Even the results of the basic test—the comparison of amylase samples from different donors—are quite impressive for the students (see below: Section 8). Nevertheless, this test setup alone is not very meaningful. However, it shows that there is an enzyme in saliva that can break down starch and that its activity varies from person to person. Different variants of the test setup make it possible to analyze some other properties of the enzyme:

5.1 | Interindividual comparison

There are multiple copies of ptyalin genes in the human genome, which also occur in different alleles in human populations. For this reason, even a small number of subjects analyzing their own ptyalin will have different ptyalin activities, illustrating the genetic heterogeneity of human populations.

5.2 | Temperature optimum

Identical sets of seven samples (Table 3) can be incubated at different temperatures (a reasonable range is between 4 and 60°C). Ensure that the respective sample sets are pretempered in heating/cooling blocks or water baths.

5.3 | pH-dependency

Different pH values are achieved with different buffer systems (see Table 4).

5.4 | Susceptibility against chemicals or detergents

Any chemicals, such as heavy metals, detergents, and so on, can be added to the reaction samples to determine their effects on enzyme activity.

6 | RESULTS

The following Figures 1–5 show the outcome of different test settings.

7 | DISCUSSION

Here, we present a didactically valuable, simple experimental setup as an example of enzyme characterization. The enzyme in question is ptyalin (salivary amylase), which is readily available and robust, and the test itself is also simple. Although the test principle is known (see, e.g., Ref. 1), its application in the variants presented here as a versatile model for enzyme characterization has not yet been described to our knowledge.

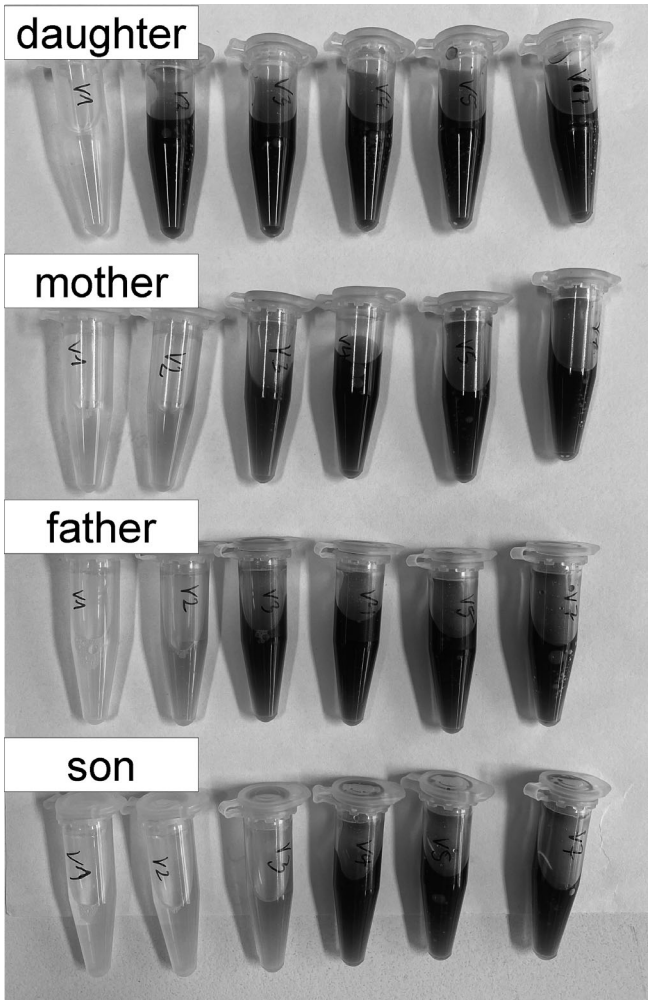

FIGURE 1 Result of a family test (Kaygusuz family). Reactions were composed according to Table 3. Four samples in the middle: Reaction samples with saliva dilution 1:25/1:50/1:100/1:200/1:400 (contrary to Table 2). First and last column: positive and negative control, respectively.

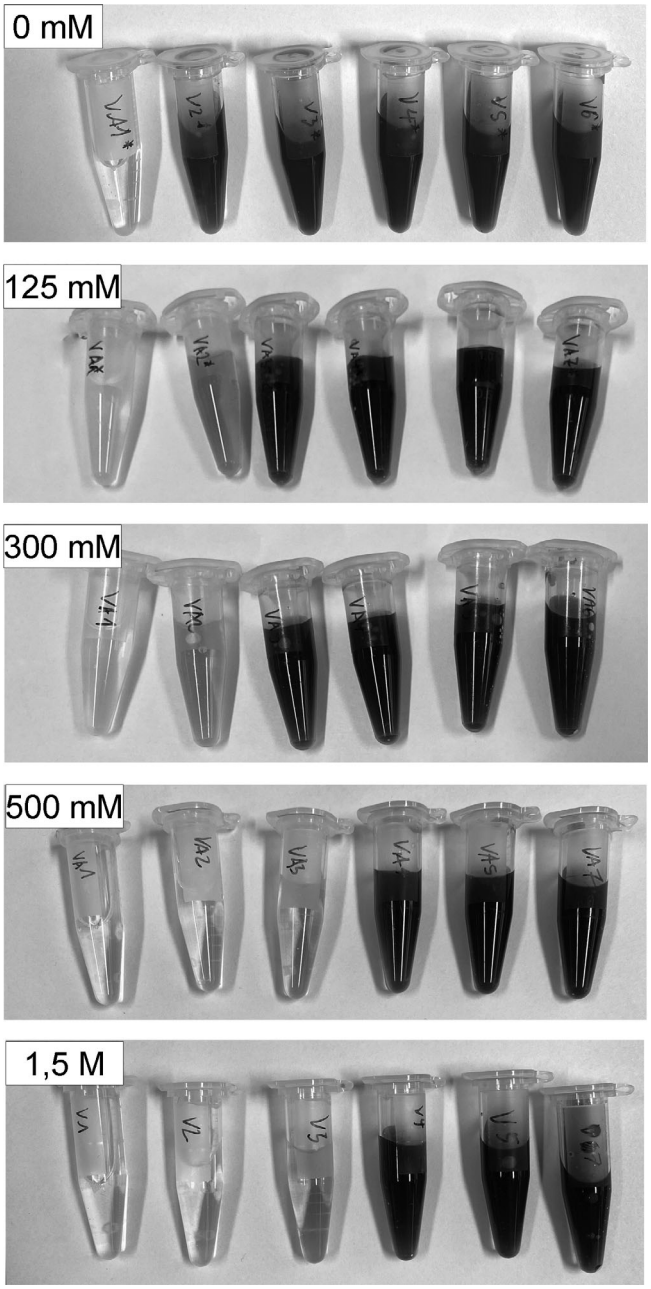

FIGURE 2 Ptyalin's dependency on Cl^-. Reactions were composed according to Table 3, however, NaCl concentrations were varied as denoted on the individual charts. First and last column: positive and negative control, respectively.

Humans have three genes for ptyalin: AMY1A, AMY1B, and AMY1C, which in turn are present in one or a few copies. There is evidence that there is an evolutionary link between copy number and the diet or dietary patterns of different human cultures, although this is still controversial.[2–5] Whatever the reason, a human individual may have only a few or even more than a dozen gene copies coding for ptyalin at a time. Therefore, the level of ptyalin activity can vary greatly from person to person. This can lead to such remarkable cases as in Figure 1 (the parents with apparently very different alleles, of which the weak ones were passed on to the daughter and the highly active ones to the son). In fact, the test of any group will give quite heterogeneous results.

Figures 2–4 show examples of test setups that are easy to interpret. The test can be extended: basically, an unlimited number of chemicals—detergents, salts, oxidizing agents, and so on—can be tested.

Exact values of enzyme activity are very difficult to determine; this would require a continuous kinetic test. Nevertheless, a rough estimate is possible. As can be seen from Figure 5, the sensitivity of the iodine–starch test is quite good. In weakly colored samples, more than 95% of the starch is digested, but a small residual amount is still present. For a rough estimate, these samples can be

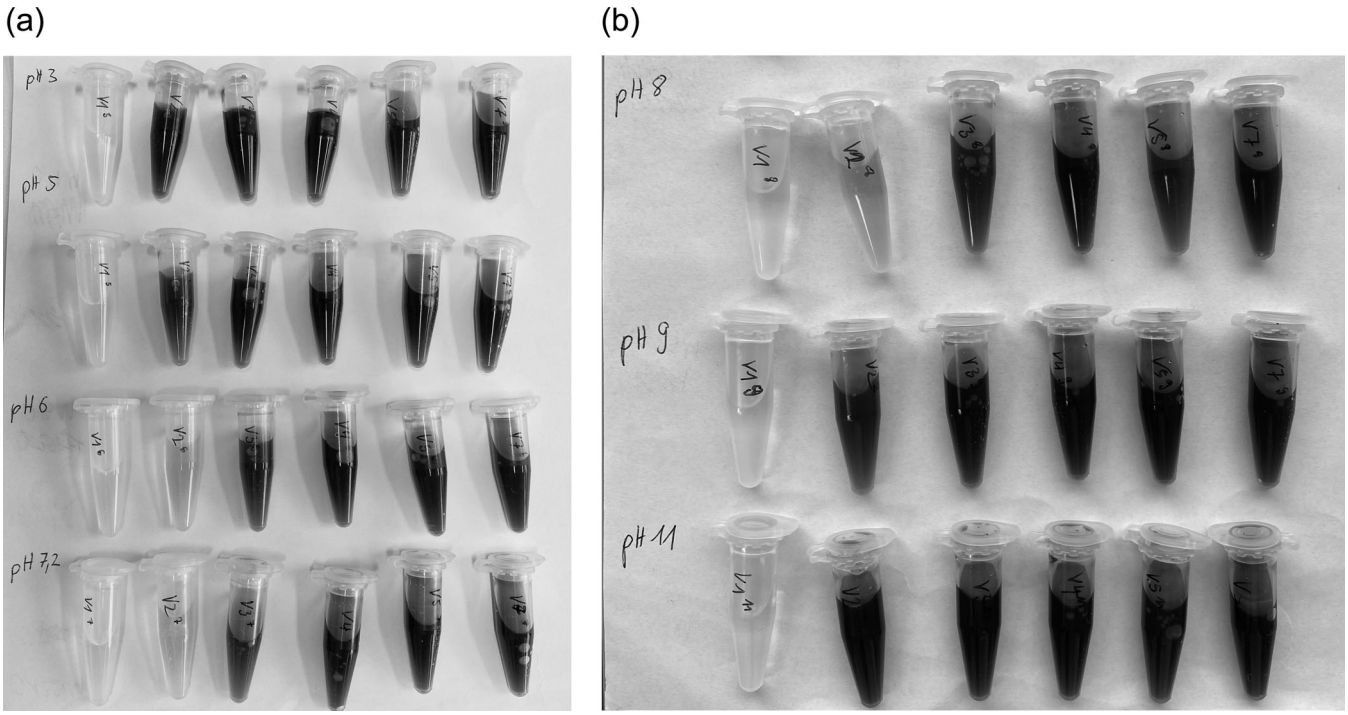

FIGURE 3 Ptyalin's pH optimum. Reactions were composed acc. to Table 3, however, pH was varied according to Table 4. First and last column: positive and negative control, respectively.

considered to have just completed starch degradation. This leads to an estimation error of about a factor of 2 or 3, but the calculated values will still fall within the correct range. Of course, topics such as the exact determination of the enzyme activity of ptyalin cannot be dealt with at school but rather in a course of study.

8 | ASSESSMENT OF LEARNING SUCCESS AND DIDACTIC COMMENTS

From 2017 to 2023, we have tested the basic version of this experiment as part of the "school lab days" at our university with a total of 57 pupils. The pupils were between 14 and 16 years old, and up to this age, the subject of enzymology is not yet covered in biology lessons in German schools. Therefore, the pupils had no real concept of enzymes and enzymatic reactions. After the experimental days, we conducted evaluations in which (a) the pupils were asked what an enzyme is and what it does, and (b) how enzymes can be characterized. About 95% (55/57) were able to answer question (a), and about 85% (49/57) knew the answer to question (b): enzymes can be characterized by their specific reaction, which is carried out and quantified under controlled and different conditions. Remarkably, the students involved in the development of the assays described here or in the supervision of the "school lab days" also reported that this experiment had considerably deepened their understanding of enzymology. (A statistical evaluation is not very meaningful due to the small number of >10). Reflection discussions with them revealed that the development of a deeper understanding of the subject of enzymology is facilitated above all by the clarity of the results: the visualization makes it much easier to develop a basic theoretical concept of enzymology. The great advantage of the experiments described here is their clearness: pupils and students can fully understand them.

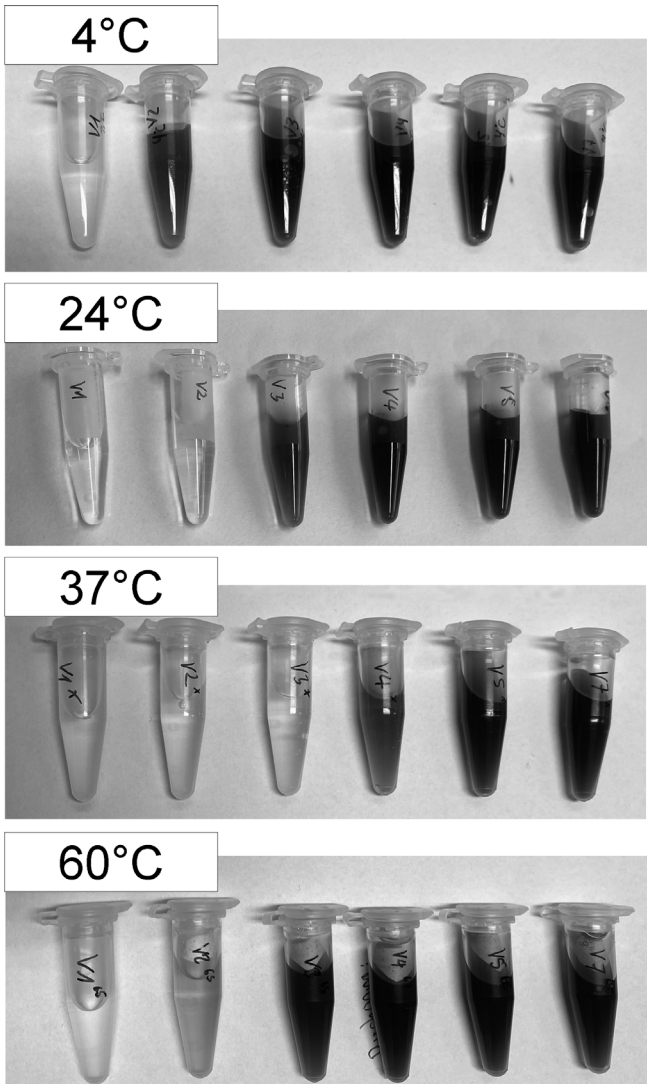

FIGURE 4 Ptyalin's temperature optimum. Reactions were composed according to Table 3. First and last column: positive and negative control, respectively. Five samples in the middle: Reaction samples with saliva dilution 1:5/1:25/1:50/1:100/1:200. Incubation occurred (from top to bottom panel) at 4, 24, 37, and 60°C, respectively.

AUTHOR CONTRIBUTIONS

Study conception and design: Andreas Beyer. Experiments and data collection: Chiara Theresa Vey, Viola Kaygusuz, and Josefa Sophia Kayser. Analysis and interpretation of results: All authors listed. Draft manuscript preparation: Andreas Beyer. All authors reviewed the results and approved the final version of the manuscript.

ACKNOWLEDGMENT

For the respective manuscript, human saliva samples were used. They were taken from the authors; all participants gave consent to the study. All data is anonymized, with the exception of the family case, for which the family has given its express consent. Moreover, the work was carried out in accordance with the university's data protection and ethical guidelines.

FIGURE 5 Test sensitivity–different amounts of starch detected with Lugol's solution. The samples contain (from left to right) 500, 100, 10, and 0 µL of starch solution (corresponding to 12, 2.4, 0.24, and 0 mg per 1 mL sample), detected with 10 µL Lugol's solution.

ACKNOWLEDGMENT

Open Access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this work are available on request from the corresponding author (Andreas Beyer).

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How to cite this article: Vey CT, Kaygusuz V, Kayser JS, Beyer A. Detection and enzymatic characterization of human saliva amylase. Biochem Mol Biol Educ. 2024. https://doi.org/10.1002/bmb.21825